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12-*O*-Tetradecanoyl phorbol-13-acetate interferes with germination of *Phycomyces blakesleeanus* sporangiospores

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The presence of protein kinase C (PKC), a key enzyme in signal transduction, has not been investigated in fungal cells. The phorbol ester TPA, an activator of PKC, may be used as an indicator of the presence and role of PKC in *Phycomyces blakesleeanus* spores. Activation of spore germination by acetate was prevented by 6 nM TPA. The TPA analog 4αPDD, an ineffective activator of PKC, did not affect spore germination. 3 mM dbcAMP, on the other hand, reversed the inhibition of germination caused by TPA. TPA-stimulated protein kinase activity was detected in spores. The possible relationship between PKC and the increased levels of cAMP that accompany the induction of spore germination is discussed.

Spore germination; Protein kinase C; Phorbol ester; cyclic AMP; (*Phycomyces blakesleeanus*)

1. INTRODUCTION

Dormant spores of the fungus *Phycomyces blakesleeanus* must be activated by heat shock or treatment with some monocarboxylic acids in order to induce their germination and growth in a suitable culture medium (review [1]). Of particular significance is the fact that the cytoplasmic levels of cAMP are transiently elevated immediately after application of activator, leading to the proposal that this second messenger might be the trigger of spore germination [2,3].

In addition to the extensively studied cAMP-generating system, the breakdown of membrane polyphosphoinositides in response to an extracellular stimulus appears to constitute another quite general mechanism for regulation in mammalian [4] and plant cells [5]. Diacylglycerol (DG), one of the two second messengers generated by such a mechanism, causes activation of a Ca^{2+}/phospholipid-dependent kinase, called protein kinase C (PKC) [6]. Increasing evidence supports the notion that PKC plays a pivotal role, as positive or negative effector, in a number of processes that include secretion, differentiation and proliferation (review [7]). Phorbol esters, like TPA, are potent tumor promoters which specifically stimulate PKC, mimicking the activating effect of DG [8]. Although PKC has been amply studied in mammalian cells, to our knowledge, no reports exist demonstrating its presence in fungi.

Here, TPA was used as a probe to examine the presence of PKC in *P. blakesleeanus* spores. Concomitantly, the potential role of this kinase in fungus germination was investigated.

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Abbreviations: PKC, protein kinase C; DG, diacylglycerol; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; 4αPDD, 4α-phorbol-12,13-didecanoate; OAG, 1-oleoyl-2-acetylglycerol; dbcAMP, dibutyryl cyclic AMP; PS, phosphatidylserine

2. MATERIALS AND METHODS

The wild-type strain NRRL 1555 (−) of *P. blakesleeanus* was used throughout this study and maintained on slants of YPG

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medium [9]. Spores were harvested as in [10]. To promote germination and growth, either the spores were heat shocked (48°C, 15 min) before inoculation or 10 mM ammonium acetate was added to the growth culture medium. Spores (10^6/incubation) were grown in 2 ml Sutter medium composed of glucose and asparagine [11] and incubated at 24°C. Stock solutions of phorbol esters and OAG were made in ethanol, which was 0.8% in the growth culture medium. Germination was determined at the indicated times and expressed as the percentage of cells exhibiting a germ tube (200 cells were counted).

PKC activity was determined in non-activated spores as follows: 1×10^9 spores were resuspended in 20 mM Tris-HCl buffer (pH 7.5), containing 0.25 M sucrose, 1 μg/ml antipain, 2 μg/ml leupeptin, 1 mM EGTA, 1 mM EDTA, and disrupted in a Braun homogenizer for 2 min with cooled CO_2. The crude extract was centrifuged ($2000 \times g$, 5 min) and the enzyme activity was measured in both the supernatant and pellet. PKC activity was assayed after 5 min incubation at 30°C of a reaction mixture (125 μl) containing 25 mM Tris-HCl (pH 7.5), 120 μg/ml histone IIIS, 5 mM magnesium acetate, 2 mM $CaCl_2$, 15 μM [γ-^{32}P]ATP (0.5 μCi/mmol), 80 μg/ml PS, 10 nM TPA and 25–200 μg spore protein. For basal ^{32}P incorporation, cofactors for PKC (Ca^{2+}, PS and TPA) were omitted from the assay medium which additionally contained 1 mM EDTA and 1 mM EGTA. The reaction was stopped by the addition of ice-cold 20% trichloroacetic acid and precipitates were collected onto Whatman paper filters which were then washed with 5 ml of 10% trichloroacetic acid, followed by 5% trichloroacetic acid. Radioactivity was measured in a Beckman LS 7800. Protein was estimated as in [12].

Materials were purchased from the following sources: TPA, 4αPDD, OAG, histone IIIS, PS and dbcAMP (Sigma); [γ-^{32}P]ATP (New England Nuclear).

3. RESULTS AND DISCUSSION

3.1. *Effect of phorbol esters on spore germination*

Fig.1 shows that 6 nM TPA counteracted acetate activation of *P. blakesleeanus* spores. Similarly, we observed 90% inhibition of germination by TPA when spores were activated by heat shock instead of acetate. Control experiments indicated, on the other hand, that non-activated spores exposed to TPA did not germinate spontaneously. The TPA concentration used in these experiments falls within the range that commonly elicits a variety of cellular responses in mammalian cells [6]. In contrast to TPA, the biologically inactive phorbol ester 4αPDD failed to block spore germination (71%). Therefore, given the remarkable specificity of TPA to activate PKC [8], the above results suggest that this enzyme can be present in *P. blakesleeanus* spores and that it might play an important role in controlling crucial processes that participate in germination. It is

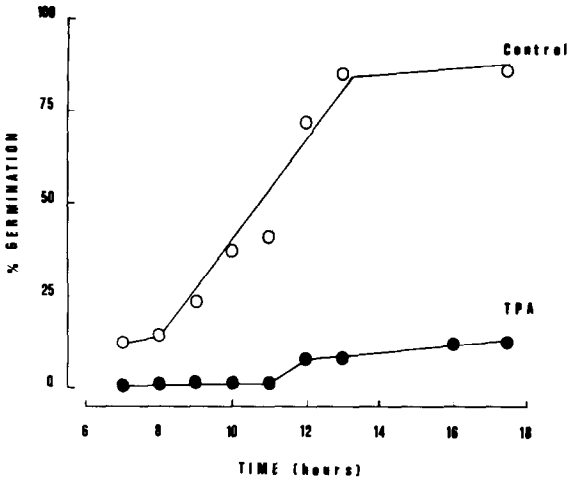

Fig.1. Effect of TPA on the time course of *P. blakesleeanus* spore germination. 10^6 spores were added to 2 ml Sutter culture medium containing 10 mM ammonium acetate and 0.8% ethanol (○) or 6 nM TPA (●). Data are the average of two independent determinations.

noteworthy that OAG, a synthetic diacylglycerol known to stimulate PKC, was unable to counteract the activation of spores (not shown). This observation can be interpreted in terms of the metabolic degradation of OAG by the spores, as demonstrated in human platelets [13]. In principle, the inhibition by TPA of spore germination could be unexpected, since the net effect of this phorbol ester on cell growth or differentiation is positive in many mammalian cells. However, biologically active phorbol esters are also capable of inhibiting cellular processes such as mitogenesis in vascular smooth muscle cells [14], progesterone secretion in ovarian granulosa cells [15], differentiation in mammary epithelial cells [16] and the responsiveness to chemoattractants in neutrophils [17].

It is not known whether growth inhibition by TPA is caused by alteration of early or late events associated with germination. Thus, we investigated the effect of 6 nM TPA, added at different times after spore activation, on the germination processes. Table 1 shows that TPA did not block the germination when added 0.25, 1, 2, 4 and 6 h after spore activation while it markedly reduced the percentage of germinated spores (15%) when present at zero time. Therefore, these results indicate that TPA exerts its inhibitory effect shortly after spore activation, presumably through stimulation of PKC. However, it is too early to ascertain the

Table 1

Effect of TPA on spore germination when added after application of activator

Period of incubation before TPA addition (h)	% germination	
	− TPA	+ TPA
0	84	15
0.25	84	80
1	90	67
3	85	85
4	83	84
6	82	85

Spores were inoculated into culture medium containing 10 mM ammonium acetate. At the indicated times, 6 nM TPA or 0.8% ethanol was added. The percentage of germinated spores was estimated at 15 h of incubation

nature of the enzymatic pathway that is affected by the activity of PKC.

3.2. *dbcAMP reverses the TPA inhibition of spore germination*

The transient elevation in the cytoplasmic levels of cAMP that occurs immediately after spore activation has been considered as one of the earlier signals that evokes germination, although little is known about the mechanism underlying the regulation of this phenomenon [2,3]. Therefore, an attractive working hypothesis is that TPA interferes, through stimulation of PKC, with the increase in cAMP levels and, consequently, inhibition of germination should occur. If this is the case, addition of dbcAMP should reverse the inhibition of acetate-activated germination produced by TPA. As illustrated in fig.2A, 3 mM dbcAMP indeed overcame the inhibitory action of TPA, eliciting a high percentage of germination (82%). In an additional experiment, spores were first incubated in acetate-containing medium plus TPA and, after 12.5 h incubation, 3 mM dbcAMP was added. Fig.2B shows that, under these conditions, dbcAMP was still capable of inducing germination (83%). Thus, PKC can be involved in regulating the resting levels of cAMP by means of inhibition of adenylate cyclase or acceleration in the rate of phosphodiesterase activity or both. For comparison, a close relationship has been observed between PKC and adenylate cyclase activities, for example, in frog erythrocytes [17] and human platelets [18].

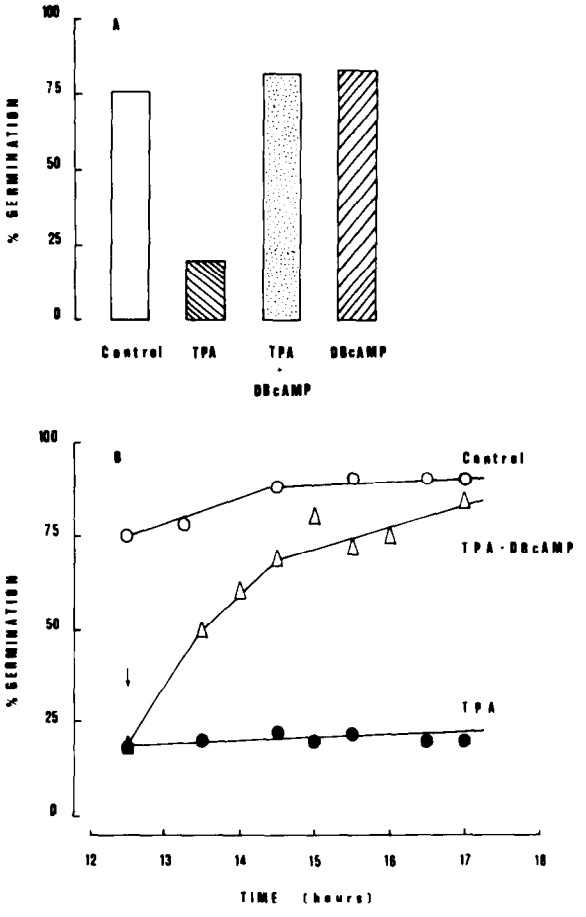

Fig.2. Reversal of TPA inhibition of spore germination by dbcAMP. (A) 10^6 spores were inoculated into 2 ml Sutter culture medium with 10 mM ammonium acetate and 0.8% ethanol (control), 6 nM TPA, 3 mM dbcAMP or 3 mM dbcAMP plus 6 nM TPA. The percentage of germinated spores was determined after 18 h incubation. Values are the average of two independent experiments. (B) Conditions essentially as in (A) except that spores were first exposed to TPA and, where indicated by the arrow, an aliquot of this spore suspension was then incubated with dbcAMP.

3.3. *PKC activity in fungal spores*

A TPA-stimulatable protein kinase was assayed in fractions from non-activated spore extracts. When measured in total homogenates, PKC activity was not detected. On the other hand, highest PKC activity, as shown by the 55% increase in kinase activity stimulated by TPA (table 2), was consistently found in the pellet obtained from a crude extract centrifuged at 2000 × *g* for 5 min. This pellet is mainly composed of spore walls and

Table 2

Protein kinase C activity in *P. blakesleeanus*

Expt	^{32}P incorporated (pmol/min per mg)		% stimulation
	− TPA	+ TPA	
A	34	55	62
B	30	45	50

PKC activity was measured as described in section 2. Each experiment was performed in triplicate

plasma membranes closely associated with them. Although inactive PKC has a cytosolic localization in mammalian cells, in our preparation this enzyme can be trapped in the pellet as a result of manipulation of the spore homogenate or be specifically bound to plasma membranes. Detailed fractionation of the cell extract will provide information with respect to this question. Basal incorporation of ^{32}P (without PKC cofactors) can be due in part to other kinases present in the spores.

In conclusion, we have demonstrated the presence of PKC in *P. blakesleeanus* spores. PKC may participate in modulating the cytoplasmic levels of cAMP associated with spore activation, although its role in other mechanisms related to the polarized growth of the spores must be considered. We are presently investigating these possibilities.

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